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| 14. ABSTRACT Rate of prostate cancer progression is affected by the reactive stroma microenvironment. Our previous studies have shown that reactive stroma is recruited to the cancer foci and functions to promote angiogenesis and tumorigenesis via TGF- β regulated pathways. The objectives of the proposed research is to assess the origin / ontogeny of reactive stroma in cancer and the fundamental mechanisms of recruitment / activation in prostate cancer. To date, we have addressed studies proposed for Tasks 1 and 2. We have developed an <i>in vivo</i> matrix trapping approach to isolate, characterize and culture reactive stromal cells recruited to Matrigel plugs. We have also developed a three dimensional co-culture model that permits co-culture of prostate carcinoma cell spheroids with prostate stromal progenitor cells. We have evaluated reactive stroma recruitment in mice that receive xenograft implants of prostate cancer cells. These studies will allow us to dissect key mechanisms that mediate recruitment of reactive stroma to the tumor microenvironment and to target these mechanisms in order to inhibit tumorigenesis. | | | | | |
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Introduction:

The tumor microenvironment is an important regulator of prostate cancer progression, however, the key mechanisms are poorly understood. The cancer-associated reactive stroma compartment is complex and the principal cell types that interact with carcinoma cells to promote tumorigenesis are not understood^{1,2}. Accordingly, although an attractive idea, the concept of targeting the tumor microenvironment with novel therapeutics has not yet been well developed. Accordingly, the purpose of this project is to deduce the origin and ontogeny of reactive stroma progenitor cells in prostate cancer and to provide proof of concept that these cells can be targeted with drug-induced gene expression. Our initial proposed hypothesis was that reactive stroma is recruited from both local and circulating progenitor cells. We now understand better the functional role and key cell types from both the local compartment and from circulating pool of cells that contribute to the genesis of reactive stroma in prostate cancer. The goal of this research is to establish whether reactive stroma recruitment to the cancer foci can be used as a novel therapeutic targeting point. The long-range goal is to target reactive stroma recruitment as a novel therapeutic for the treatment of prostate cancer in order to disrupt the biology of the reactive stroma niche and therefore alter the progression of the cancer.

Body:

Task 1. Task 1 was to complete the proposed Specific Aim 1 studies as follows: "To determine mechanisms of local reactive stroma recruitment using a novel matrix trapping procedure and to assess the role of TGF- β in driving both recruitment and activation to reactive stroma phenotype."

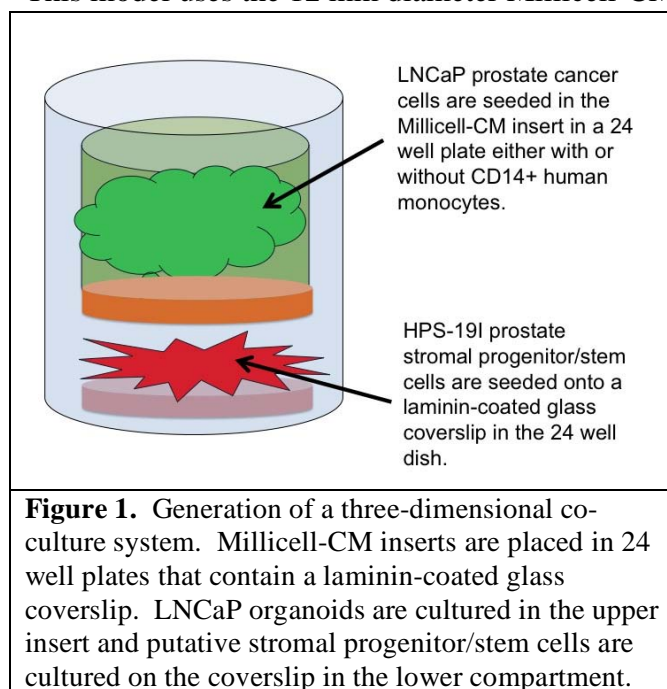
The purpose of this Aim is to refine the matrix trapping procedure outlined in the application in order to define the population of cells recruited to reactive stroma. In addition, we have also worked to establish a novel co-culture model as proposed for this Aim. The purpose was to use the co-culture model to deduce the mechanisms of progenitor or reactive stroma cell interactions with human prostate cancer cells.

This Task/Aim has been essentially completed and we have developed an improved, second-generation co-culture model from which to address mechanisms. Our initial matrix trapping studies used EHS Matrigel plugs inoculated subcutaneous into the flanks of NCr *nu-nu* (nude) mice as originally proposed. Matrigel plugs were removed, analyzed by light microscopy and immunohistochemistry, and used as a source to culture trapped stromal cells that invaded the Matrigel in vivo. As reported in the previous Progress Report, mouse stromal cells (vimentin positive) were recruited to the Matrigel plugs and have been successfully cultured and subpassaged. Additional experiments to characterize their phenotype and capability as putative multipotent stromal stem cells are being planned during the next review period. We have been successful in culturing these cells and will be able to use the procedures developed to probe for specific biological mechanisms of cell fate determination and recruitment mechanisms as proposed. We anticipate the evaluation of these cells in the three-dimensional co-culture model as described below once we have fully-optimized this system to address mechanisms.

As discussed in the previous Annual Report, a second aspect of this Task/Aim has been the development of a co-culture model system that can be used to evaluate interactions between stromal cells and cancer cells to assess key cell types and mechanisms that will include the role of TGF-beta. To expand on this aspect of Aim 1, we have used the HPS-19I human prostate stromal cell line in a second-generation co-culture model. There are many advantages to an in vitro approach such as easy manipulation of cells, defined media conditions, and the ability to sort out cells at the end of an experiment to evaluate gene expression patterns in a cell type specific manner.

The HPS-19I human prostate stromal cell line was chosen for several reasons. We initiated this cell line from normal prostate tissue (no histopathological evidence of prostate disease) derived from a cadaver donor. The cell line was continuously cultured in media Bfs following protocols we have published previously³⁻⁶. Our initial flow cytometry analysis showed this cell line to be positive for both CD90 and CD44 suggesting the cell line may function as a progenitor/stem cell for the evolution of reactive stroma. Hence, use of this cell line was deemed to be ideal to evaluate the mechanisms of local reactive stroma recruitment and to address the regulators of reactive stroma formation, including the role of TGF-beta.

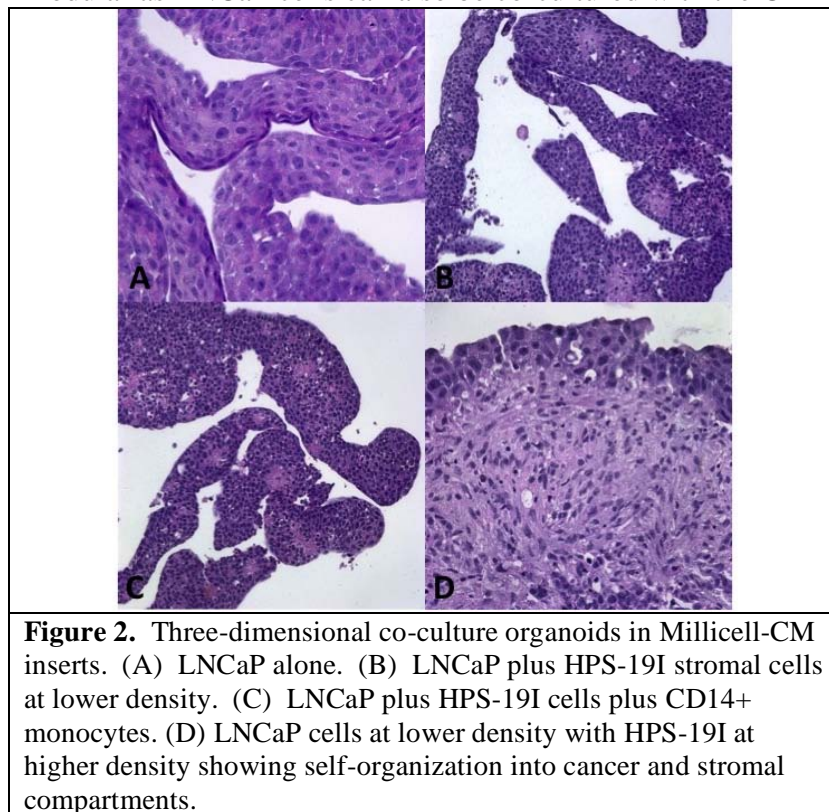
A second generation, two-dimensional co-culture model was developed as shown in Figure 1. This model uses the 12 mm diameter Millicell-CM membrane (0.4 μ m pore) insert (Millipore) in



24 well tissue culture plate fitted with 12 mm diameter PolyD-Lysine/Laminin Cellware coated glass cover slips (BD BioCoat). The HPS-19I cells were seeded on the Laminin coated coverslips and LNCaP human prostate cancer cells alone or together with human CD14 positive monocytes seeded in the upper chamber Millicell-CM insert. An advantage of the Millicell-CM insert is that cells do not attach to the membrane and cancer cells form a free-floating organoid in this chamber. All cells were seeded in their respective chambers in serum-containing media and allowed to incubate 24 hr. and then switched the cultures to serum-free, chemically-defined media (DMEM plus high glucose, 0.2% bovine serum albumin, insulin, transferrin, selenium, plus

penicillin/streptomycin) for 72 hr. Respective cultures were then analyzed for phenotype (including FACS analysis) to assess differentiation and gene expression profile analysis to assess potential mechanisms. We have generated preliminary data as outlined below.

Figure 2 shows that human prostate cancer LNCaP cells form free floating organoids in the upper Millicell-CM insert under these fully-defined media conditions. The model is very modular as LNCaP cells can also be co-cultured with the CD14 cells or with the HPS-19I cells in the upper insert chamber.

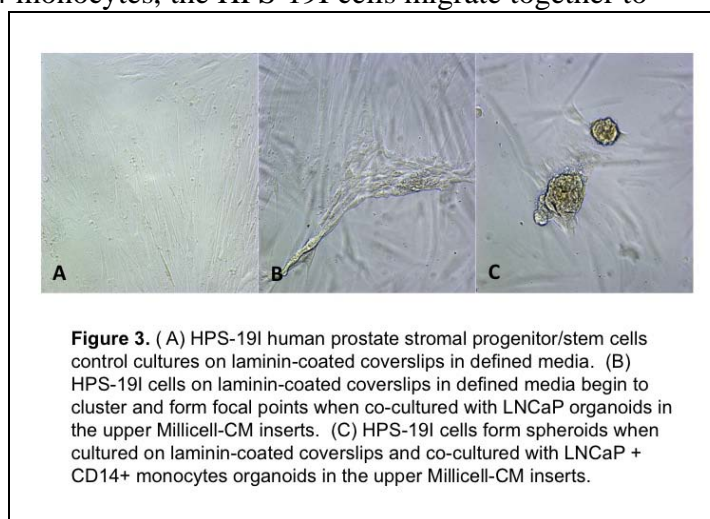


Under these conditions, the LNCaP cells, HPS-19I stromal cells and the CD14 monocytes grow together as a continuous organoid where the HPS-19I derived stromal cells form an interior position with CD14 cells integrated into the organoid. We will use immunohistochemistry to confirm differentiation status of cells in studies planned during the next year of support.

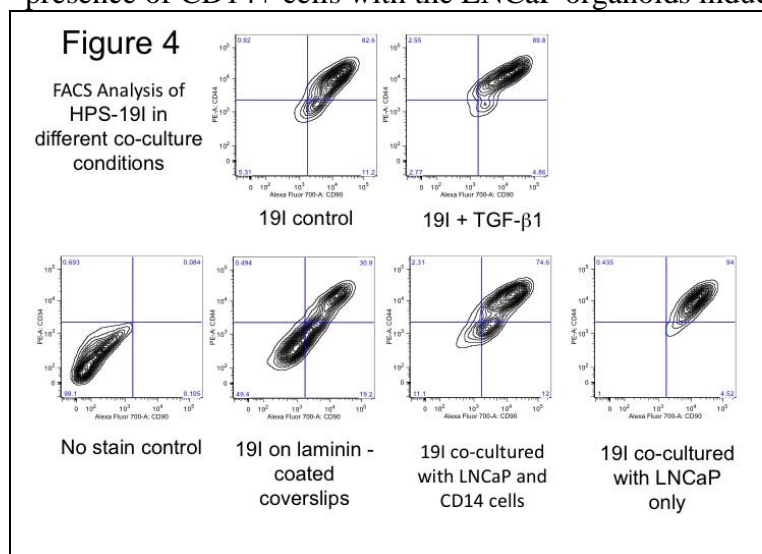
Figure 3 shows the phenotypic changes in the HPS-19I cell line when these putative stromal progenitor/stem cells are cultured on the lower chamber Laminin-coated coverslip and exposed to different LNCaP organoid

conditions in the upper Millicell-CM insert chamber. The HSP-19I cell line alone on the coverslip forma a typical fibroblastoid monolayer. When co-cultured with LNCaP organoids these cells seem to migrate and form monolayer foci. Of interest, when co-cultured with organoids composed of LNCaP plus CD14 monocytes, the HPS-19I cells migrate together to form three-dimensional spheroids, a feature associated with progenitor/stem cells.

This cancer/monocyte-induced change in HPS-19I phenotype is also associated with altered FACS analysis as shown in Figure 4. When cultured alone these cells will differentiate and proceed from double positive (CD90+/CD44+) to double negative cells. When cultured with TGF-beta (50 pM), these cells are maintained as double positive. When cultured on the laminin coated coverslips, these cells differentiate to double negative cells more rapidly, however when co-cultured on laminin coverslips and with



LNCaP cancer organoids these cells are maintained in the double positive status. Of interest, the presence of CD14+ cells with the LNCaP organoids induced the HPS-19I cells to resume a trend



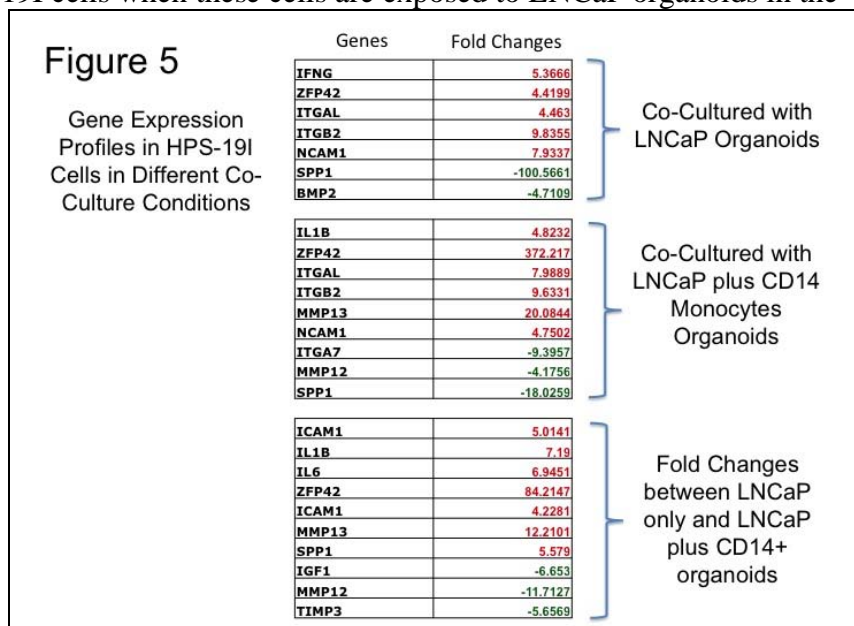
toward double negative status. We are in the process of repeating these studies and will analyze additional markers of differentiation and phenotype.

Our preliminary studies also show that the changes in phenotype and FACS profile corresponds with differential gene expression in HPS-19I cells as shown in Figure 5. Replicate experiments and quantitative PCR are currently being conducted to verify results. Of interest, these data shows that

expression of the zinc finger binding protein ZFP42 gene is elevated 4 fold when the cells are co-cultured with LNCaP organoids and is elevated 372 fold when cells are co-cultured with LNCaP plus CD14 cell organoids. In addition, several other notable chemokines such as interferon gamma and interleukin 1B are altered. Also of interest, the expression of osteopontin (SPP1) is greatly downregulated in HPS-19I cells when these cells are exposed to LNCaP organoids in the upper chamber. This is of interest since osteopontin has emerged as a key matricellular regulator of inflammation, stromal cell reactive biology and of cancer cell invasion. Current studies are directed to verify gene expression using quantitative PCR.

Since we have a LNCaP cell line engineered to overexpress TGF-beta, we plan to also use these cells in the three-dimensional culture model to address how TGF-

beta affects the differentiation of putative stromal stem cells. We have not yet determined the overall effects of overexpression of TGF- β 1 in this model, however in our preliminary studies to date with the first generation co-culture model shows that there appears to be larger LNCaP spheroids in these co-cultures (shown in the previous Annual Report). Overall, this data is important, as the new co-culture method will allow us to examine co-cultures of recruited reactive stromal cells together with organoids of human LNCaP carcinoma cells in fully defined



media conditions where growth factors or agents that inhibit certain signaling pathways can be added in order to dissect mechanisms of interactions as planned.

In summary, we have expanding key experiments and approaches in Task1/Aim1 in order to complete this Task using the most relevant cell types. We have made considerable progress on Task1/Aim 1 goals and anticipate completing these studies in the final year of the project.

Task 2. Task 2 is to complete Specific Aim 2 studies: To determine the mechanisms of reactive stroma recruitment from marrow-derived circulating progenitors and to assess the role of TGF- β in active recruitment and induced differentiation to reactive stroma.

We have completed the adoptive transfer experiments and have conducted additional bone marrow transplant studies to evaluate the contribution of bone marrow derived cells to the genesis of reactive stroma in the DRS LNCaP/Matrigel xenograft system as proposed. To date, we have completed three adoptive transfer experiments using human bone marrow cells (CD34 positive and CD14 positive) as part of another funded study. Even though these experiments were not part of this project, we have gained considerable experience at successfully completing adoptive transfer of blood borne cells into the tail vein of nude mice. We have also successfully isolated circulating progenitor fibrocyte cells (pro-fibrocytes) from mouse peripheral blood and have cultured these. We have also isolated and successfully cultured eGFP labeled murine bone marrow stromal cells as shown in Figure 6. We anticipate using these cells in the three-dimensional co-culture model described in Aim 1. We have completed bone marrow transplant

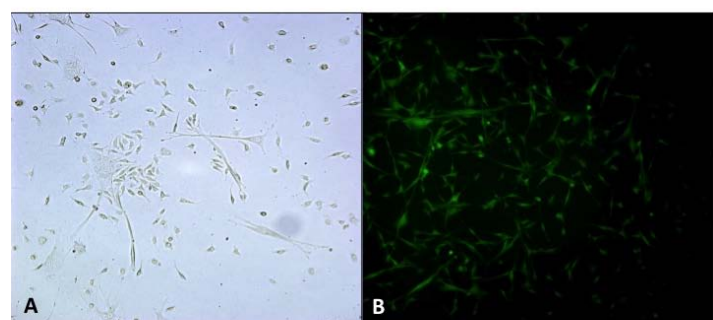


Figure 6. Cultures of adherent marrow-derived murine cells were established from bone marrows of eGFP (enhanced green fluorescence protein) mice. Cells demonstrate a fibroblastoid morphology (A) and express eGFP (B).

studies using engineered mice as marrow donors with transplants into irradiated NCr *nu-nu* mice. Again, this was funded as part of another study. These studies were important since they were used as a backup approach to address the central question of what is the relative contribution of either bone marrow derived cells or resident stem/progenitor cells in the co-evolution of reactive stroma at sites of cancer foci.

Key to the present study, the marrow transplants studies have shown that bone marrow derived cells are recruited to sites of developing reactive stroma. However, these studies have shown that cells do not likely differentiate to myofibroblasts or carcinoma-associated fibroblasts. Instead, these cells appear to be monocyte-like cells and may differentiate to dendritic cells and/or macrophage population. Our studies are ongoing and we plan to fully characterize the evolution of these cells in the tumor microenvironment. These results were the impetus for designing the second generation co-culture model. We plan to use the co-culture method to further assess the role of TGF-beta in this differentiation process. No other significant changes in the nature or scope of the plans in the Statement of Work are expected for Task 2.

Task 3. Task 3 will be to complete Specific Aim 3 studies: To use a drug-inducible expression system to assess whether progenitor cells can be targeted to deliver drug-induced gene expression at sites of recruitment / activation of reactive stroma and whether this approach can uncouple key recruitment pathways.

Our current plan is to initiate Task 3/Aim 3 studies as soon as we have fully characterized and verified the differentiation status and gene expression profiling in Task 1 and Task 2 studies. During the final year of support, we will work on engineering the putative progenitor/stem cells with drug-inducible gene expression as proposed. We plan to engineer the HPS-19I cells first and will follow with the murine derived marrow cells. Next we will proceed with Task 3/Aim 3 studies as proposed. No significant changes in the nature or scope of the plans in the Statement of Work are expected for Task 3.

Key Research Accomplishments:

- Development of an *in vivo* matrix trapping procedure that allows for phenotype characterization of recruited reactive stroma in situ and for isolation of these cells for cell culture.
- Development of a first generation *in vitro* co-culture method that uses LNCaP spheroids combined with prostate stromal cells in fully defined and serum-free culture media.
- Development of protocols to isolate and culture circulating murine pro-fibrocyte cells, murine bone marrow stromal cells and human CD14+ monocytes.
- Development of a second-generation *in vitro* and three-dimensional co-culture method that permits modular inclusion of several cell types in direct contact with LNCaP organoids or in a separate chamber and cultured on coverslips with different attachment factors in fully defined media.
- Demonstration of key changes in putative stromal progenitor/stem cell differentiation and gene expression profiles that is affected by both prostate cancer cells and CD14+ monocytes.
- Demonstration that recruitment of bone marrow derived cells to LNCaP xenografts using adoptive transfer and bone marrow transplant studies. These studies suggest that these cells are monocyte-like cells that do not differentiate to reactive stroma myofibroblasts or carcinoma-associated fibroblasts.
- Key data that suggests reactive stroma is likely derived from resident stromal progenitor/stem cells that are influenced to differentiate via interactions with cancer cells and recruited bone marrow derived monocyte/macrophages. These data help to pinpoint these cells as the targets for drug-induced gene expression to be done in Task 3 studies.

Reportable Outcomes:

- Presentation of this data at several seminars and national meetings by Dr. Rowley.
- Some of this data was used to support the proposed use of the co-culture methodology in several other research proposals. These have been submitted to the NIH, the DOD and to the Cancer Prevention Research Institute of Texas (CPRIT).

Conclusions:

We have made key progress on Tasks 1 and 2 and are nearing completion of these studies. We plan to prepare and submit a manuscript in the next 6 months. Task 3 is actually the most straightforward Task and will be completed before the end of the project. We have considerable experience in engineering stromal cells^{5,6} once the key target cell has been identified and evaluated in Tasks 1 and 2. Hence, we expect to be able to complete the engineering of drug-inducible gene expression as proposed in Aim 3. Importantly, this work has led to development of a novel co-culture method that recombines human prostate carcinoma cell spheroid/organoids with human prostate stromal progenitor/stem cells in a three dimensional culture model that is maintained in serum-free conditions. We believe these studies will allow us to pinpoint the key cell type(s) that co-evolve as reactive stroma in prostate cancer and to assess the importance of TGF- β signaling to this recruitment. Moreover, we should be able to target this cell with inducible gene expression as a proof of concept that targeting the reactive stroma microenvironment is possible. It is anticipated that these studies will allow us to develop critical pre-clinical data from which to base a strategic approach aimed at targeting the reactive stroma in human patients.

It should be reiterated that little is understood about the mechanisms of how reactive stroma generates and from what cell progenitor this reactive stroma is derived from. These are critical questions to be addressed if targeting the microenvironment is ever to emerge as a novel therapeutic approach. The present study remains on target and we anticipate the generation of at least two key publications will result from this work.

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RE: Annual progress report

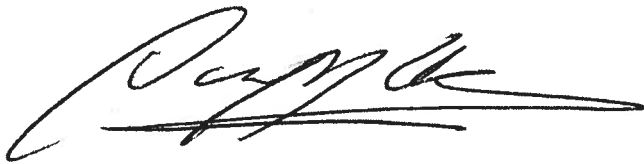
To whom it may concern:

Attached, please find the progress report for:

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It covers the period 25 Feb 2009 - 24 Feb 2010.

Sincerely,



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